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PARTIAL CHARACTERIZATION OF AN ALKALOPHILIC EXTRACELLULAR CRUDE PECTINASES FROM A BACILLUS POLYMYXA STRAIN

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تعريف جزئي لانزيم البكتنيز القلوي لبكتيريا الباسيلس بوليمكسا

ايهـاب الرجبـي وعـادل محاسـنه

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لقد اختيرت عزلة بكتيرية مكونة للأبواغ من جنس الباسيلس من تربة أم قيس بسبب قدرتها على إنتاج إنزيمات البكتنيز المفرزة خارج الخلية وتم تعريفها للنوع باسيلس بوليمكسا وتبين أن هذه العزلة تنمو بصورة مثالية على درجة حرارة ٣٧°م ورقم هيدروجيني قدره ٧ وانها قادرة على انتاج العديد من الانزيمات المفرزة خارج الخلية مثل انزيم البروتينيز والاميليز والسيلوليز اضافة لانزيم البكتينيز وتم دراسة انزيمي البكتين ليبز والبكتيت ليبز واتضح انهما ينتجان بصورة مثالية بعد ١٢و١٥ ساعة على التوالي في مرحلة النمو اللوغرتمي. وعند تحديد صفات انزيم البكتين ليبز الخام تبين ان فعاليته المثالية تحدث على درجة حرارة ٣٠°م ورقم هيدروجيني قدره ٨,٥ في حين انها لانزيم البكتيت لايز ٣٠°م ورقم هيدروجيني ٩.

أما من حيث المركبات النازعة مثل EDTA فقد ادى استعمال ١ مليمولر لفقد ما يقارب من ٤٨٪ من فعالية انزيم البكتيت ليبز في حين ان مركب NTA أدى لفقد ما يقارب من ٥٣٪ لهذا الانزيم و٣٥٪ لانزيم البكتين ليبز، أما أثر بعض العناصر الثقيلة على فعالية هذه الانزيمات فقد لوحظ الأثر المثبط للكوبالت والزئبق والنحاس على انزيم البكتين ليبز عند استعمالها بتركيز ١ ميلمولر في حين أن المنغنيز والمغنسيوم وتحت نفس التركيز اثرا بصورة ايجابية لرفع فعالية هذا الانزيم بحدود ١٠٥ و١٤٠٪ على التوالي.

أما أنزيم البكتيت ليبز فقد تم تثبيطه بالكوبالت والزئبق والنحاس والحديد وتحت نفس التركيز أعلاه في حين أن المغنسيوم والزنك والمنغنيز فقد اثرا بصورة ايجابية لرفع فعالية هذا الانزيم بحدود ١٠٦ و١٨٦ و٢٣٠٪ على التوالي.

Keywords : Alkalophillic pectinases, charecterisation, *Bacillus polymyxa.*

ABSTRACT

A mesophilic spore forming isolate from the soil of Um-Qais region, which could produce extracellular pectinases was tentatively identified as *Bacillus polymyxa*. This bacterium grew best at 37°C and pH 7.0 and was able to produce extracellular enzymes other than pectinases, e.g.: protease, amylases, and cellulases. The production of two pectolytic enzymes, pectin lyase and pectate lyase, by this organism was investigated. Pectin lyase production reached its maximum in 12h cultures, while pectate lyase reached its maximum production in 15h cultures.

The optimum pH and optimum temperature for pectin lyase activity were 8.5 and 30°C, respectively, while for pectate lyase the optima were 9 and 30°C repectively. Chelating agents such as 1mM EDTA caused a decrease in pectate lyase activity by about 48%, nitrilotriacetate (NTA) also caused a decrease in activity of pectin lyase and pectate lyase by about 35% and 53% respectively. Divalent ions such as, Co^{+2}, Hg^{+2}, Cu^{+2}, and Fe^{+3} inhibited pectin lyase activity at the concentration of 1mM, while Mg^{+2}, and Mn^{+2} stimulated the enzyme activity to about 105% and 140% respectively at the same concentration. Pectate lyase, was inhibited by Co^{+2}, Hg^{+2}, Cu^{+2} and Fe^{+3} at the concentration of 1mM, while Mg^{+2}, Zn^{+2}, and Mn^{+2} stimulated its activity to about 106%, 186%, and 230%, respectively at the same concentration.

INTRODUCTION

Microbial pectolytic enzymes play an important role in plant pathogenicity, symbiosis, decomposition of plant deposits, digestion of plant foods, retting, fruit and vegetable spoilage involving rotting, and in other fermentation industries [1,2]. These enzymes are the core for a number of industrial processes including retting of flax and other vegetable fibers, extraction, clarification and depectinization of fruit juices [3], extraction of vegetable oils and maceration of fruits and vegetables [4]. A number of such enzymes from mesophilic and thermophilic bacteria and fungi have been purified [5,6].

The depolymerizing enzymes pectin lyase and pectate lyase usually have a high optimum pH values (> pH 8.5) and act by β-eliminative mechanism of attack on their substrates resulting in formation of products with a double bond between C-4 and C-5 conjugation of the double bond with the carboxyl group at C-5 brings about maximum absorption at 235 nm [7,8]. In this work we report on partial characterisation of two pectolytic enzymes from a newly isolated strain of *Bacillus polymyxa*.

Materials and Methods

Isolation of Bacteria

Samples of soil, water, and organic materials including juice wastes, plant parts and manure, were collected from various locations in Jordan. These samples were used for isolation of bacteria producing extracellular pectinases.

Aliquots (0.1 ml) of the suitable dilutions in sterile medium were plated onto pectin agar medium plates [9] containing (g / 100 ml [0.1 yeast extract, 0.5 pectin, 0.01 $MgSO_4$, 0.001 $FeCl_3.6H_2O$, 0.02 NaCl, 0.02 $CaCl_2.2H_2O$, 0.05 K_2HPO_4, 1.5 Agar), pH of the medium was adjusted under sterile conditions to 7.0 after autoclaving. Incubation temperature was 37±1°C.

Plates prepared from enrichment cultures, and that contained well separated colonies were flooded with a solution of polysaccharide precipitant cetavlon (cetyl trimethyl ammonium bromide) [9], 1% (w/v) of the reagent was dissolved in a 15% alcoholic solution, and was then used to detect pectinase production. After 20-30 minutes exposure, bacterial colonies producing pectinase (s) were

identified by the production of clear zones against an opaque color of the non-hydrolyzed medium. Colonies with clear zones were subcultured onto new pectin agar plates and were then tested again to establish and confirm pectinase production.

Characterization and identification of the isolate.

Morphological and biochemical properties were investigated using 15h cultures growing on pectin agar (PA) plates. The pectolytic mesophilic isolate was identified following conventional methods for the presumptive identification of endospore forming gram positive rods [10].

Optimization of growth conditions.

Determinations of the optimum pH, temperature, salinity, pectin, starch, cellulose, and casein hydrolysis were done according to accepted standards [11].

Pectolytic enzyme assay.

Two types of depolymerase enzymes were assayed for activity.

1. Pectin lyase

Pectin lyase activity was assayed in 50mM Tris amino-methane Tris HCL buffer (pH8.2), with 1mM Ethlenediaminetetraacetic acid (EDTA), and 0.2% pectin [12]. Two ml of 0.2% pectin solution was incubated with 0.5 ml of crude enzyme in a total volume of 5ml with buffer, and then the reaction mixture was incubated at 30°C for 20 minutes. The reaction was terminated by heating in boiling water for 10 minutes [13].

The absorbance at 235 nm was measured using Perkin-Elemer spectrophotometer (Hitachi 200, Japan). One unit of the enzyme was defined as the amount of enzyme which increases an optical density of 0.01 at 235 nm/minute corresponding to the formation of 1μmol of unsaturated uronide/minute [14].

2. Pectate lyase.

Pectate lyase activity was asayed in 50 m MTris-amin - methane Tris HCL buffer (pH 8.5), with 1mM $CaCl_2$, and 0.2% pectic acid [15]. Two ml of 0.2% pectic acid solution was incubated with 0.5 ml of crude enzyme in a total volume of 5ml with the buffer, and the reaction mixture was incubated at 30°C for 20 minutes [16]. The reaction was terminated by heating in boiling water for 10 minutes.

The absorbance at 235 nm was measured using a Perkin-Elemer spectrophotometer (Hitachi 200, Japan). One unit of the enzyme was defined as the amount of enzyme which increases an optical density of 0.01 at 235 nm/minute corresponding to the formation of 1(mol of unsaturated uronide/minute [17].

Effect of nitrogen and carbon sources on pectinase production.

Each earlnmeyer flask (300ml) containing 100ml of the medium composed of (g/1000ml, 0.2 yeast extract, 0.2 NaCl, 0.02 $MgSO_4.7H_2O$, 0.417 KH_2PO_4, 0.33 K_2HPO_4, pH 8.1) was supplemented with the appropriate nitrogen or carbon source (0.2% w/v). The flasks were inoculated with 1ml of a 15h bacterial culture grown in pectin glucose medium, and incubated for 24h at 37±1°C on an orbital shaker, 300rpm.

Crude pectolytic enzyme characterization.

Optimum pH and temperature.

The optimum pH for activity of the enzyme was determined by incubating the reaction mixture (0.5 ml of crude enzyme solution, 2ml substrate, and 1ml of either 5mM EDTA or $CaCl_2$) in addition to suitable buffer at final pH values of 4, 5, 6, 7, 8, 8.2, 8.5, 9, and 10. The mixtures were incubated at 30°C for 20 minutes, and the enzyme activity was assayed as described. The optimum temperature was determined by incubating the enzyme mixture (0.5 ml crude enzyme solution, 2ml of the substrate, and 1ml of either 5mM EDTA or $CaCl_2$) with the Tris-HCL buffer of 50mM capacity, at temperatures (10, 20, 30, 40, 50, 60, and 70°C) for 20 min, and the enzyme activity was assayed as described.

Thermostability and stability at different pH values.

The crude enzyme solution was incubated at different temperatures (10, 20, 30, 40, 50, 60, and 70°C) for 1h and the enzyme activities were determined as described above. Simultaneously 1ml of the crude enzyme solution was incubated with 4ml of buffer preparation at a final buffering capacity of 50mM at 4°C for 6h. The enzyme activities were then assayed.

Effect of chelating agents and metal ions.

Solutions of the chelating agents (EDTA), and Nitrilotriacetate (NTA), and divalent metal ions ($BaCl_2$, $MgSO_4$, $CaCl_2$, $MnCl_2$, NaCl, $CoCl_2$, $FeSO_4$, $HgCl_2$, $CuSO_4$, and $ZnSO_4$) were used. An aliquot of 0.5 ml of the crude enzyme solution, 2ml of substrate, 1ml of EDTA or $CaCl_2$ were mixed to give 1mM as a final concentration, and 1ml of Tris-HCl buffer with 50mM capacity of pH 8.5 or 9.0, was incubated under optimum temperature (30°C) for 20 minutes with different metal ions or chelating agent with a final concentration of 1mM and a final mixture volume of 5ml. The enzymatic activities were then determined by the standard method.

Results

Morphological characteristics of the isolate.

The cells of the parent isolate (SP) that was grown on nutrient agar medium were aerobic, motile, rod like single (dominant) or pairs, spore former (subterminal, oval spores). The isolate belongs to the genus *Bacillus*. Colonies of the isolate were white, circular, entire, flat, and smooth.

The isolate was cultured on a medium containing peptone, starch, and neutral red, the results showed tentatively the characteristics of *Bacillus polymyxa.*

Optimization of growth conditions.

Optimum pH

The minimum pH for growth was 6.0, where an optical density (O.D) of 0.52 was measured as indicated in Fig. 1, at pH 5 and 4, a very poor growth was detected, where the optical densities of 0.06 and 0.05 were recorded respectively. The maximum pH was 8.5 and an O.D of 1.63 was recorded. The optimum pH for growth was 7.0, where the highest O.D (2.43) was observed.

Optimum temperature.

As shown in Fig. 2, there was no growth at less than 25°C nor at more than 45°C, the highest optical density obtained (2.40) was at 37±1°C. The minimum and maximum temperatures of growth for the parent strain was 25°C and 45°C, respectively.

Optimum NaCl concentration.

Growth of the parent isolate (SP) in the medium containing various concentrations of NaCl is shown in Fig. 3. The highest O.D (2.13) was recorded in the presence of 0.2% (w/v) NaCl. Abundant growth was also obtained in the presence of 0.1% (O.D 1.92) and 1% (O.D 1.71) (w/v) NaCl. The isolate was unable to tolerate more than 2% NaCl, where no growth was recorded after 24h of incubation.

Growth and pectinases production under optimal conditions.

Maximum pectin lyase activity (67.40 μ/ 0.5ml) was detected after 12h growth (Fig. 4), while the maximum pectate lyase activity (69.10 μ / 0.5 ml) was detected after 15 h of growth. However, the optical density of the isolate (SP) at 50 nm was highest (3.07) after 15 h of growth (Fig. 4). The pH of the medium decreased gradually from the initial value of 7.0 to 6.83 after 9 h of growth, and then the pH of the medium began to increase at the end of the exponential phase of growth to reach 7.17 after 24 h.

Characterization of crude pectinases.

Effect of pH.

The pectolytic activity dependance on pH is illustrated in Fig. 5. The optimal pH of the pectin lyase activity was 8.5 with pectin as a substrate, while the optimal pH of the pectate lyase activity was 9.0, at the standard assay condition as mentioned in the "Materials and Methods" section.

The pectin lyase activity was sensitive to acidity, only

28.5% of the total enzyme activity was detected at pH 4.0. However, the enzyme was able to tolerate alkaline condition with 85.7% of the total enzyme activity at pH 10. The pectate lyase activity was also sensitive to acidity with only 10% was detectable at pH 4.0. However the enzyme was able to tolerate alkaline condition with 99.2% of activity still measured at pH 10.

As shown in Fig. 5, pectin lyase was almost 100% stable at pH 7.0, whereas at pH 6, 5, and 4, a sharp decrease in stability occurred. Above pH 8.2, a sharp decrease in stability also occurred reaching only 70% of the activity at pH 10. The pectate lyase was almost 100% stable at pH 7.0, below and above this value, a sharp decrease in stability occurred with only 34.5% and 56% of activity maintained at pH 4.0 and 1, respectively.

Optimum temperature and thermostability.

Maximum pectin and pectate lyase activities were obtained at 30°C (100%) after incubation of the reaction mixture for 20 min at pH 8.5 and 9.0 in the presence of 1 mM EDTA and 1 mM $CaCl_2$, respectively (Fig. 6). At 70°C, the pectin lyase and pectate lyase activities decreased to only 48.9%, 57% of the maximum value obtained at 30°C, respectively.

As shown in Fig.6, the pectin lyase retained about 100% of its activity after heat treatment for 1h at the temperature range of 10-30°C, while pectate lyase retained about 100% of its activity after heat treatment for 1h at the temperature range of 10-40°C. Both pectolytic enzymes investigated retained about 70% of their activity after heat treatment at 70°C for 1h.

Effects of chelating agents and metal ions.

The pectolytic activity of the crude enzymes in the presence of chelating agents and metal ions was determined and the results are presented in Fig. 7. For pectin lyase activity, final cation concentrations of 10^{-3} M; Mg^{+2}, Ca^{+2} and Na^{+} had a slight stimulatory effect on the enzyme activity, Mn^{+2} at 10^{-3} had slight stimulatory effect reaching 141% of the total control activity, whereas Zn^{+2} and Ba^{+2} at the same concentration had a slight inhibitory effect. The ions Fe^{+2}, Hg^{+2} and Cu^{+2} at a concentration of 10^{-3} M had an inhibitory effect with only 25.2%, 15.5% and 5.0% of the total control activity respectively. The enzyme was inactivated by 1mM Nitriloacetate (NTA) and retained only 65.7% of the total control activity.

For pectate lyase, a final cation concentration of 10^{-3} M of Mg^{+2} had a slight stimulatory effect, whereas Mn^{+2} and Zn^{+2} at the same concentration had a stimulatory effect on the enzyme activity to about 200% and 186% of the total control activity , respectively. At a final concentration of 10^{-3} M, Ba^{+2} and Na^{+} a slight inhibitory effect was observed with only 0.0%, 36.8%, 38.8%, and 24.2% of the total control activity detected. The cation Ca^{+2} was particularly effective in stimulating the activity. The enzyme was also inactivated by 1 mM of NTA and retained only 47.4% of the total control activity.

Cell growth and enzyme production under optimal conditions.

The parent isolate (SP) was cultivated in pectin peptone broth under optimal growth conditions (pH 7.0, 37±1°C and aeration rate of 300 rpm). Pectin lyase was assayed at pH 8.5, whereas, for pectate lyase, the pH of the reaction mixture was adjusted to 9.0. Maximum pectin lyase activity (105.00 μ / 0.5 ml) was detected after 12h of growth (Fig. 8), whereas, maximum pectate lyase activity (98.00 μ / 0.5 ml) was measured after 15 h of growth. However, the optical density of the isolate *(SP) at 550 nm was 2.98 after 18 h of growth (Fig. 8).

Discussion

Bacillus sp. strain SP which was tentatively identified as *Bacillus polymyxa* was able to grow in nutrien salt medium supplemented with pectin as the sole carbon source, suggesting that this strain is a pectolytic bacterium. Such substrates usually require more than one pectolytic enzyme for their hydrolysis [18, 19]. Other authors reported the isolation of aerobic spore forming bacteria that were

capable of producing several extracellular pectinolytic enzymes [1, 20]. Work on bacterial pectolytic enzymes from *Erwina* species and others was prompted by the belief that some of these enzymes may be a virulence determinant [21]. Optimization of growth and growth environmental conditions of strain SP showed best growth at 37°C and at an initial pH of 7.0 shaking at 300 rpm. Chesson and Conder [22] and Ward and Fogarty [23] reported the isolation of different pectolytic strains of *Bacillus subtilis* with optimal growth at 30°C, pH 7 and 150 rpm. Pectolytic enzyme production may vary greatly with medium composition [6]. In this study two pectolytic enzymes from the strain SP were investigated: Pectin lyase and pectate lyase which were produced constitutively but maximum enzyme production was observed when pectin was the sole carbon source in the medium. Nagel and Wilson [24] reported a simillar observation with *B. polymyxa* grown on media containing pectin and pectate as the sole carbon source. Nasser et al. [15] found that a thermophilic *Bacillus sp.* was able to produce endopectate lyase only when pectin was in the medium indicating its inducible manner of production. The production of pectin lyase and pectate lyase in this study indicates that such enzymes have a nutritional function. This is unlike scavenger enzymes that are secreted at the end of the active growth [25] and is similar to most extracellular enzymes secreted by *Bacillus* species [26]. Peptone was the best nitrogen source for pectinase production and cell growth. The initial pH for pectin lyase and pectate lyase production was 7-7.2 which was also the optimum pH for the growth of the SP isolate. These results are simiar to the pattern described for *Bacillus polymyxa* by Nagel and Wilson [24] and *Bacillus subtilis* by Ward and Fogarty [23]. No significant pH differences were observed during the growth experiments irrespect of the carbon sources available. Maximum production of pectin and pectate lyases by *Bacillus polymyxa* strain SP occured at the late exponential phase (12-15h). This result is similar to that reported by Priest [2] and Nasser et al. [15]. The optimum pH for pectin lyase and pectate lyase production by *Bacillus* strain SP were 8.5 and 9.0 respectively. Rombouts and Pilnik [1] had similar results on a *Bacillus subtilis* strain. Reportedly both enzymes were relatively active and stable at high temperatures and alkaline pH values. Initial catalytic characterization of the crude pectin lyase in this work indicated that this enzyme was inhibited by 1 mM Co^{+2}, Zn^{+2}, Fe^{+2}, Cu^{+2} and Hg^{+2} but was not affected by Ca^{+2}, Ba^{+2}, and Na^{+}. The cations Mn^{+2} and Mg^{+2} had a stimulatory affect, but Ca^{+2} at a final concentration of 10^{-3} M had no effect on both enzymes. However, Mn^{+2}, Zn^{+2} and Mg^{+2} were somehow stimulatory to pectate lyase at the concentration of 10^{-3} M whereas EDTA, Co^{+2}, Hg^{+1}, Fe^{+2}, Cu^{+2}, Ba^{+2}, Na^{+1} and NTA were inhibitory. Ikeda et al. [27] and Tanabe et al.[17] reported similar results on Bacillus pectin and pectate lyases.

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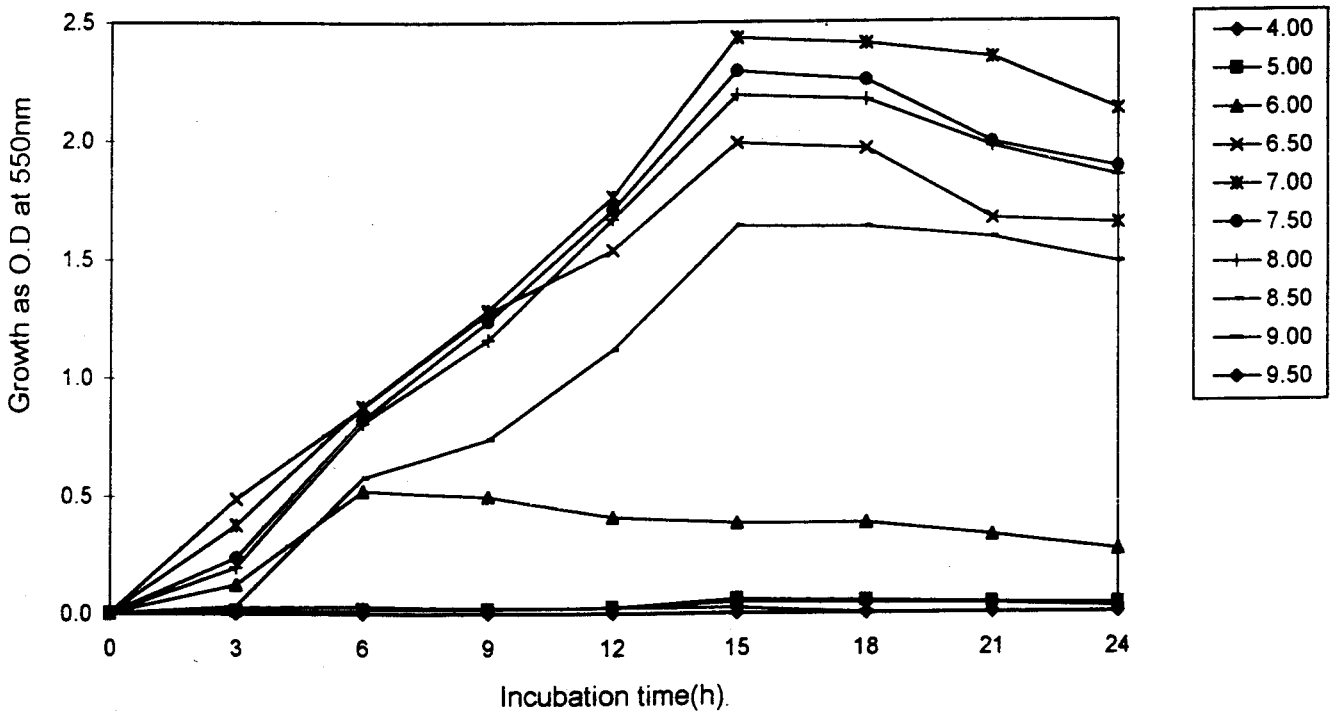


Fig. 1 : Determination of the optimum pH for growth of the parent strain SP.

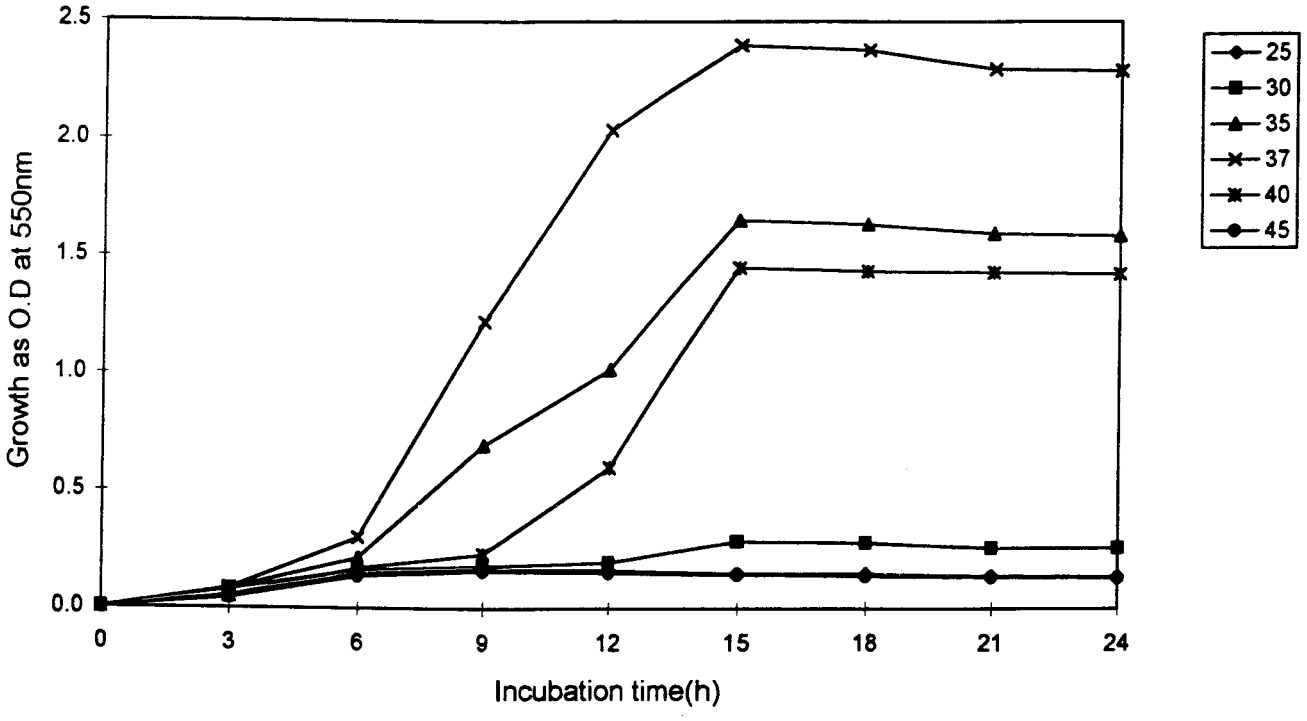


Fig. 2 : Determination of the optimum temperature for growth of the partent strain SP.

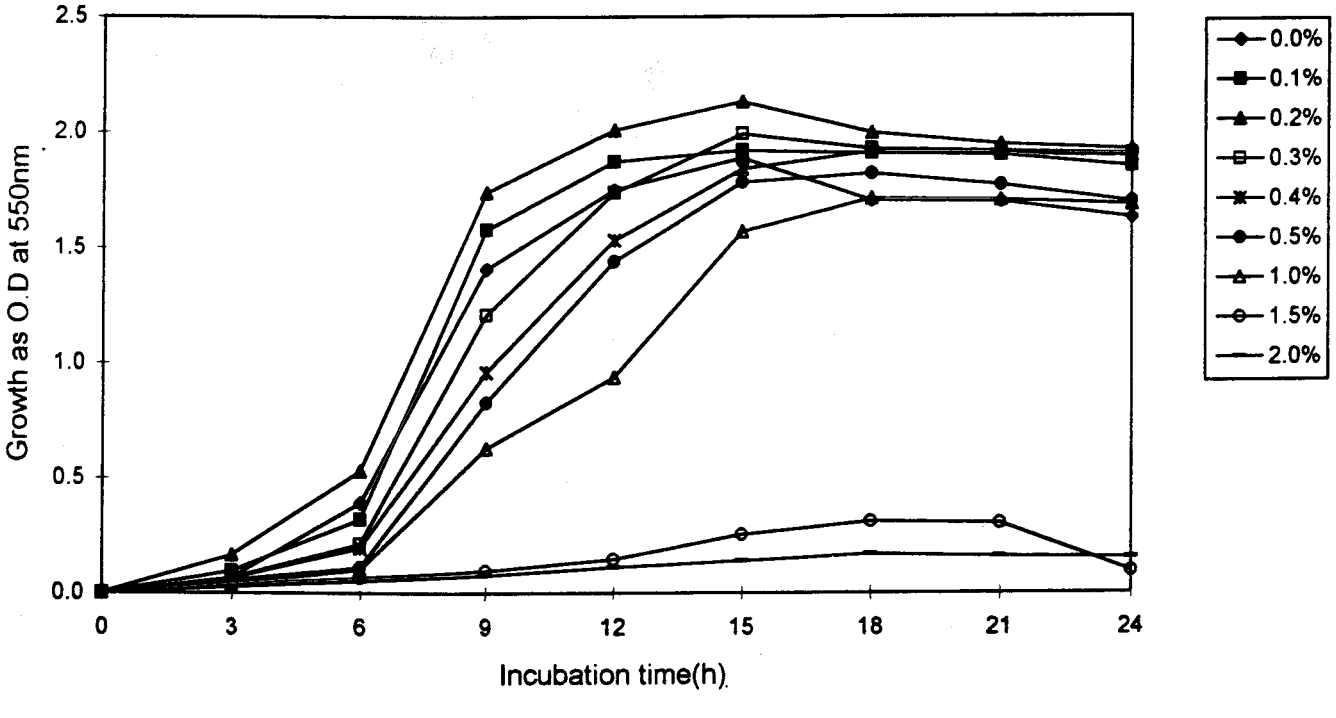
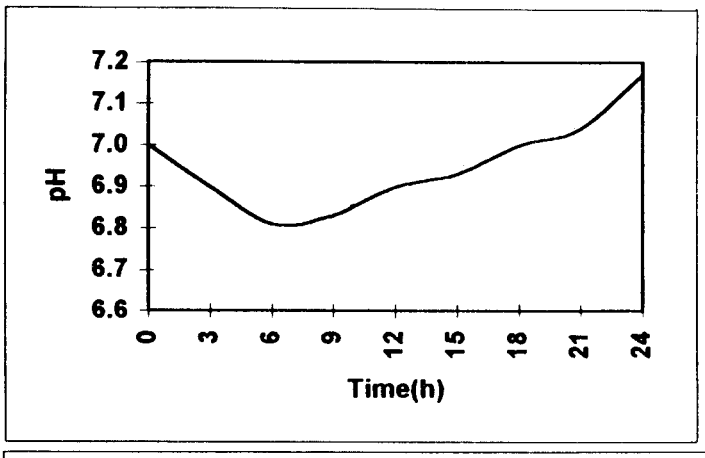
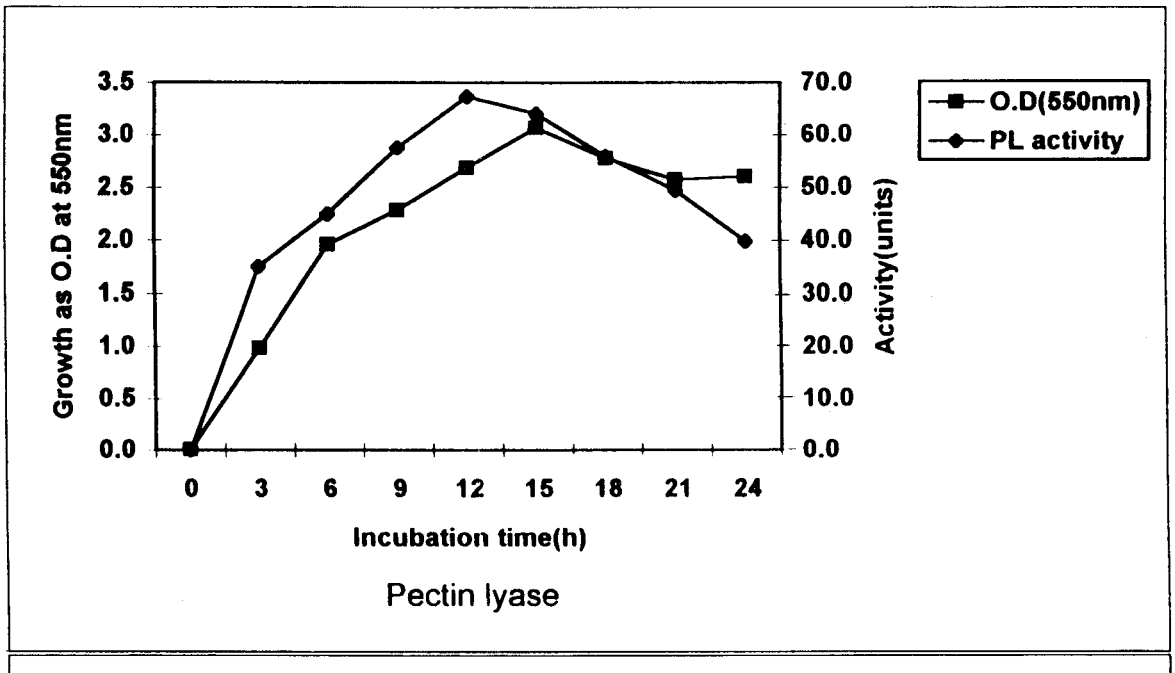
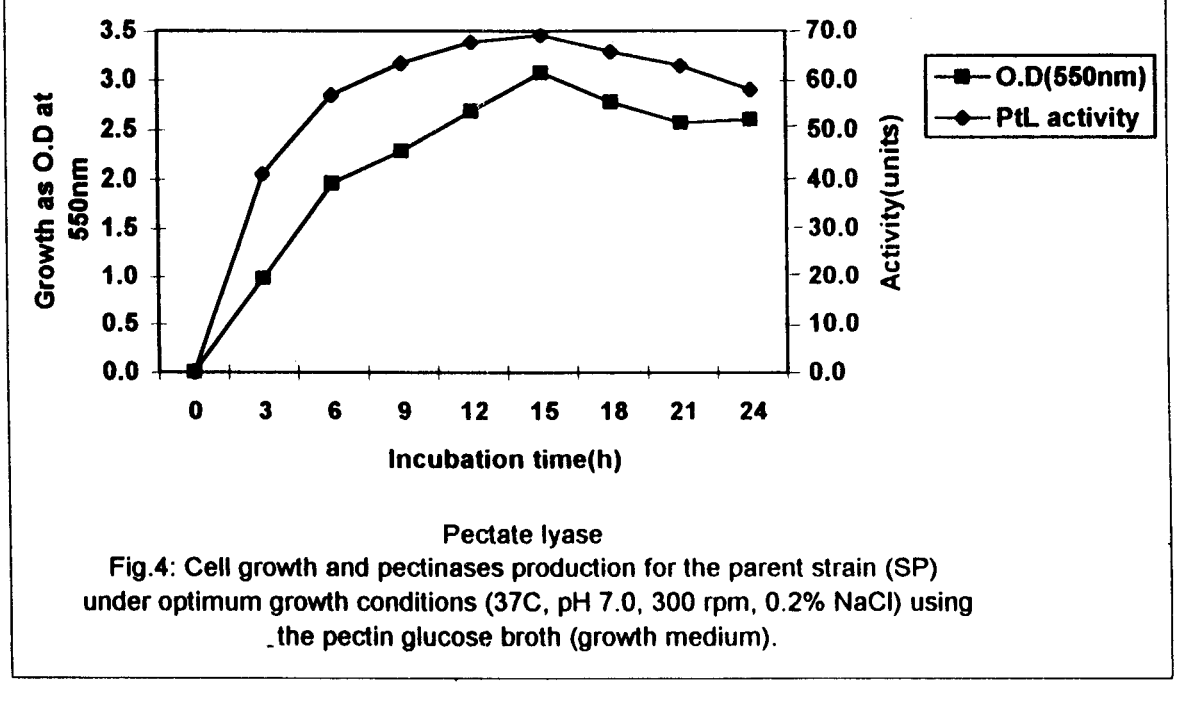


Fig. 3 : Determination of the optimum NaCI concentration for growth of the parent strain SP.



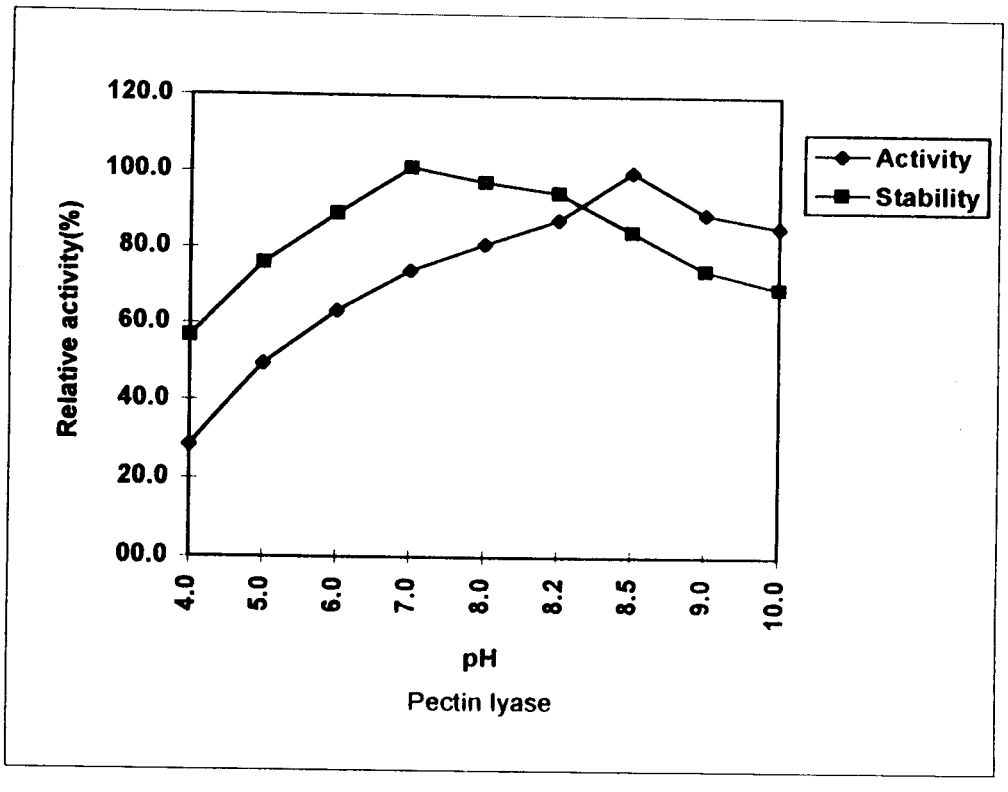


Pectin lyase



Pectate lyase

Fig.4: Cell growth and pectinases production for the parent strain (SP) under optimum growth conditions (37C, pH 7.0, 300 rpm, 0.2% NaCl) using the pectin glucose broth (growth medium).



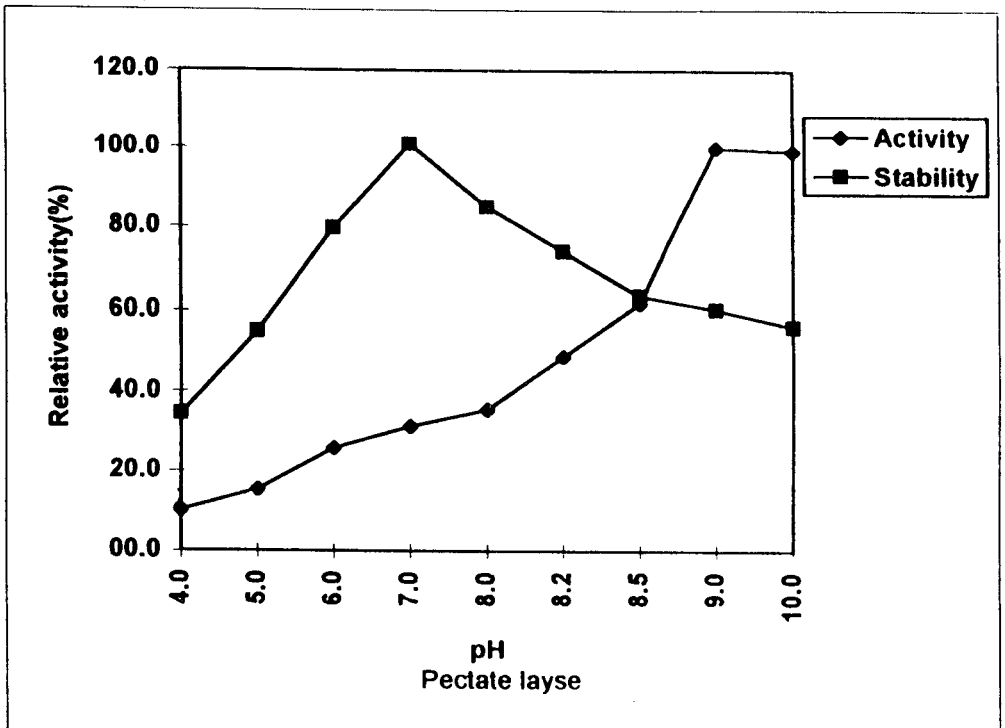
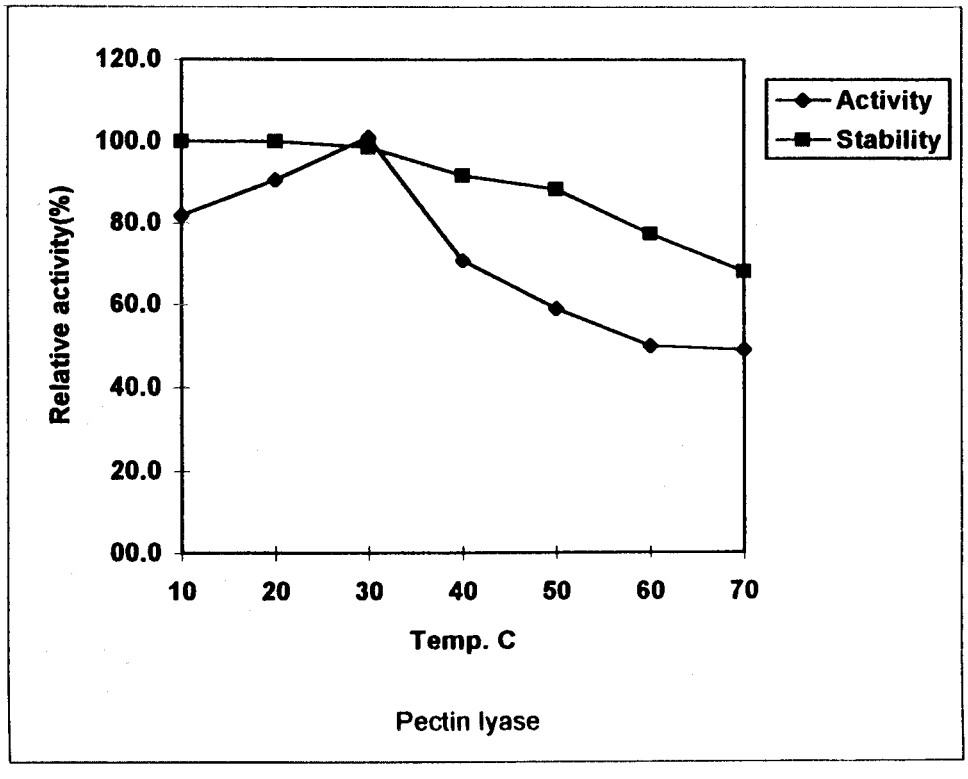


Fig. 5 : Effect of pH on activity and stability of the pectinases from the partent strain (SP).



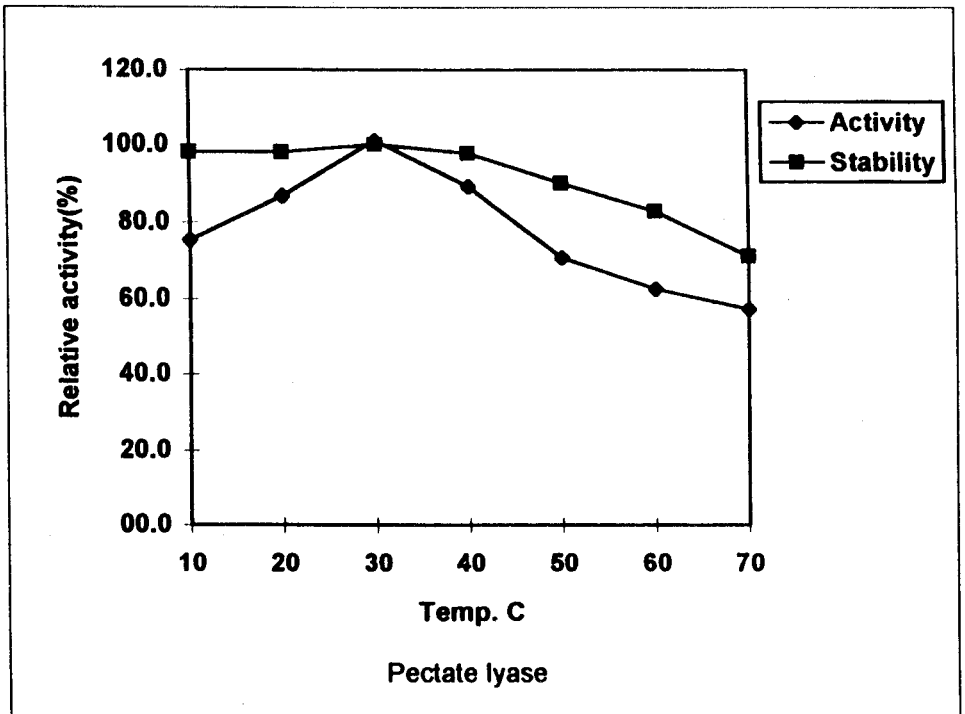
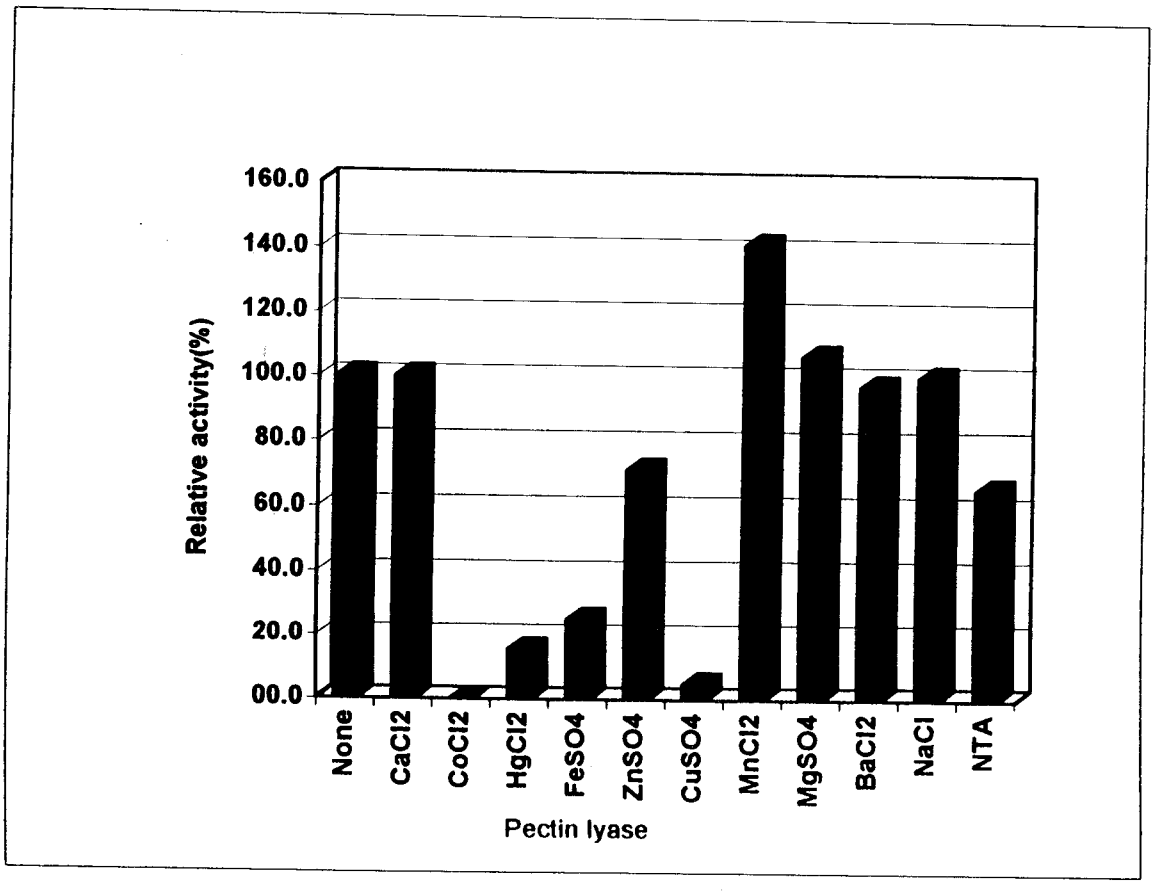


Fig.6: Effect of temperature on activity and stability of the pectinases from the parent strain (SP)



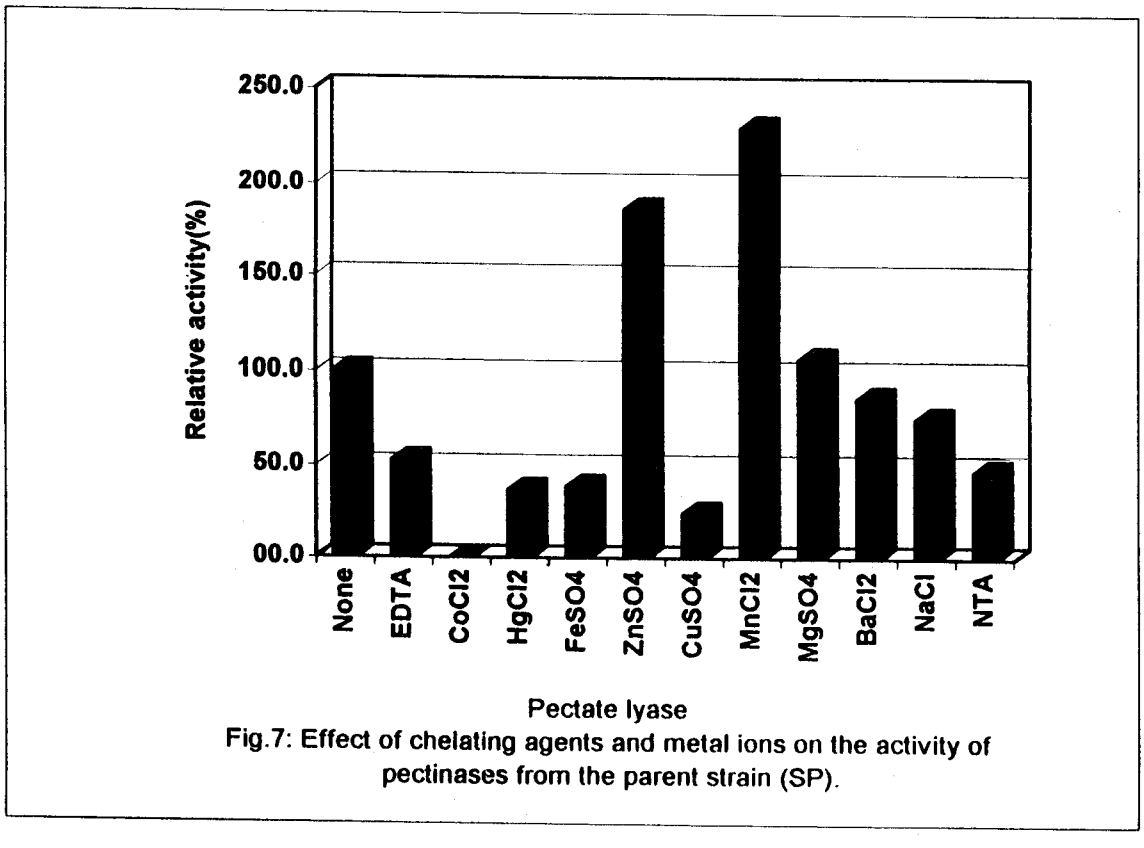
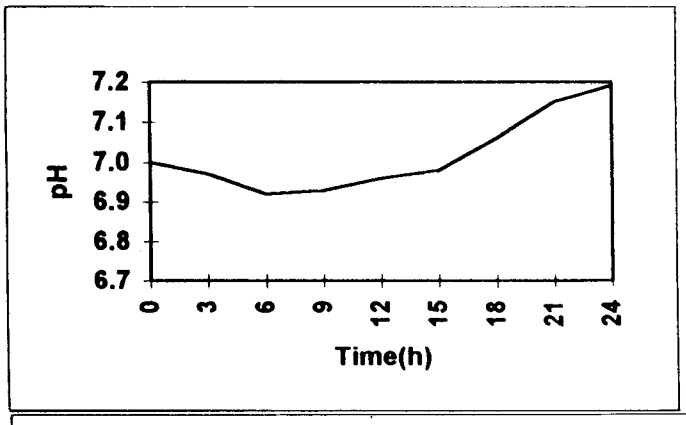
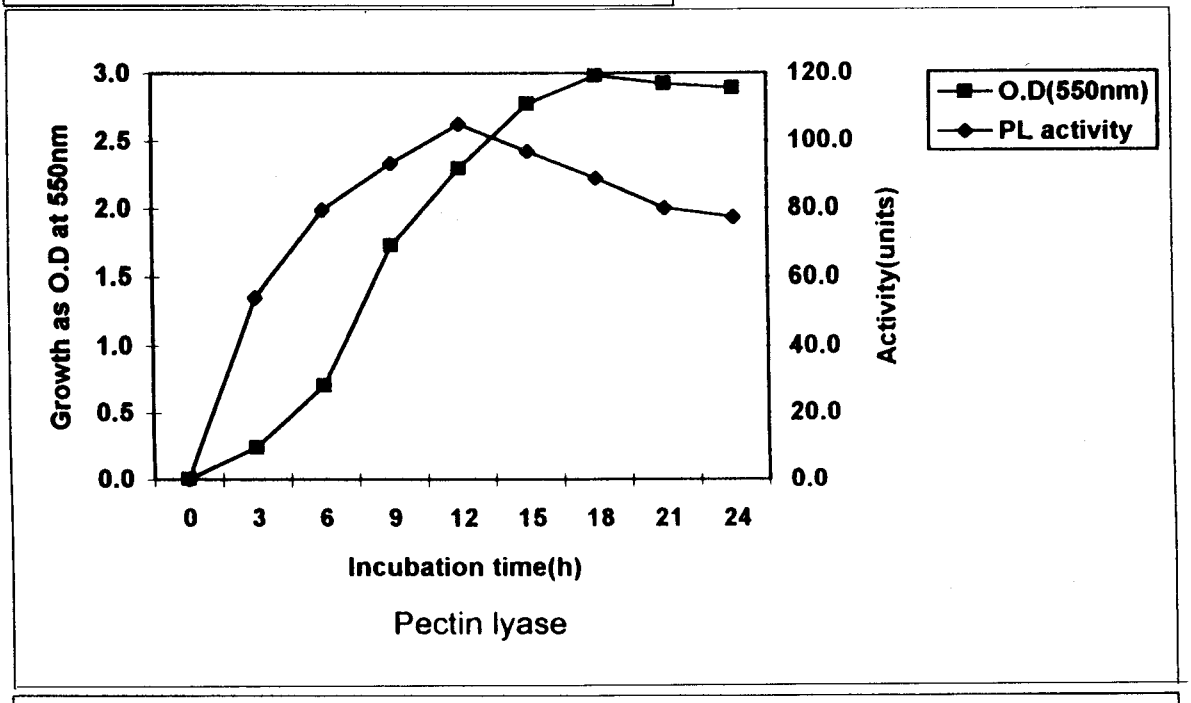
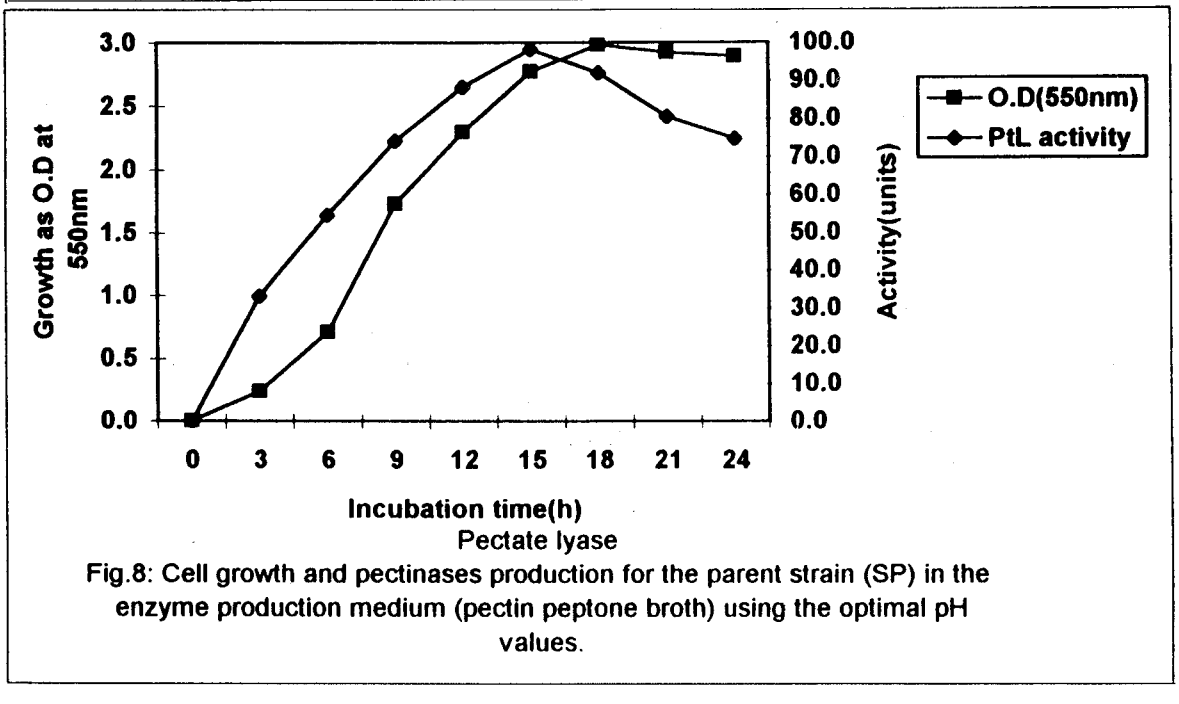


Fig.7: Effect of chelating agents and metal ions on the activity of pectinases from the parent strain (SP).





Pectin lyase



Pectate lyase

Fig.8: Cell growth and pectinases production for the parent strain (SP) in the enzyme production medium (pectin peptone broth) using the optimal pH values.